# MIB-1 Proliferative Activity Is a Significant Prognostic Factor in Primary Thick Cutaneous Melanomas

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**Although the Breslow measurement of tumor thickness of melanoma is the most significant predictor of survival, the biologic behavior of thick lesions remains unpredictable. MIB-1, a monoclonal antibody to a Ki-67 epitope, recognizes all proliferating cells. Unlike Ki-67 antibody, which requires frozen tissue, MIB-1 can be used on formalin-fixed tissue. Proliferation, measured by MIB-1 expression and mitotic index, was assessed as a prognostic factor in a group of patients with clinical stage I thick cutaneous melanoma (tumor thickness 4 mm or greater), for which predicted survival is low. From a melanoma data base, 97 patients with this type of melanoma were identified. Of these, 64 had lesional tissue available for study. The median follow-up time was 3.8 years (range 0.42–13.6 years). The percentage of MIB-1 reactivity was scored as low at less than 10% (n = 33), intermediate at 10% to 20% (n = 17), and high at greater than 20% (n = 14). Melanomas with high MIB-1 reactivity were associated with significantly poorer cause-specific survival compared with tumors with intermediate ($p < 0.0001$) or low MIB-1 reactivity ($p = 0.0025$). Multivariate analysis demonstrated that MIB-1 reactivity was a significant independent prognostic factor related to cause-specific survival ($p = 0.0002$) and was more sensitive than tumor thickness or mitotic index in this select group of high-risk patients. Identification of individuals with stage I thick cutaneous melanoma who are at risk of recurrent disease may improve patient management as new therapeutic modalities become available.** ***Key words: proliferation/antibody/immunohistochemistry. J Invest Dermatol 105:22–26, 1995***

Over the past 30 years, the incidence and mortality rates of melanoma have increased dramatically. At the same time, our knowledge of the biologic behavior of melanoma has been furthered by the studies of Clark *et al* [1,2]. Prognosis is directly related to the depth of invasion in the dermis [2,3], and the single most important predictor of survival is the thickness of the melanoma measured from the granular layer of the epidermis to the deepest portion of the tumor [3]. Although other pathologic features such as the nature of the lymphocytic infiltrate, ulceration, regression, mitotic rate, and vascular invasion may add prognostic information [4–6], melanoma remains an unpredictable disease. Deaths have been reported in patients with thin melanomas of less than 0.76 mm, a vertical measurement that was originally thought to be associated with a virtual cure [7]. Even thick melanomas have a variable prognosis. At least 50% to 75% of patients with melanomas 4 mm thick or greater will die of their disease within a few years; the remaining 25% to 50% of patients survive for unknown reasons [7]. Other factors may be important in determining the clinical outcome of this challenging disease.

Manuscript received November 30, 1994; final revision received March 6, 1995; accepted for publication March 16, 1995.

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Abbreviation: PCNA, proliferating cell nuclear antigen.

The mitotic rate or index, identified as an independent prognostic factor in melanoma by Clark *et al* [4], is calculated on hematoxylin and eosin–stained sections and expressed as the number of mitotic figures per 10 high-power fields or per 1.0-$mm^2$ area [8]. Although this is easily performed, tissue section thickness and subjectivity may confound the determination of the mitotic rate or index. Furthermore, mitoses represent only a small portion of the growth cycle, whereas a proliferation antigen such as Ki-67 is expressed throughout the growth phase of the cell cycle ($G_1$, M, $G_2$, and S, though not in $G_0$) [9,10]. The proliferative rate measured with the Ki-67 antibody has been suggested to be valuable in predicting the prognosis of melanoma [11,12]. Immunohistochemistry with the Ki-67 antibody can be performed only on frozen tissue, however, and studies using this antibody thus have been limited by the availability of fresh tissue and by a relatively short follow-up period [13]. Proliferating cell nuclear antigen (PCNA) is another proliferation marker that has been used in assessing tumor aggressiveness or prognosis of melanocytic neoplasms [14–17]. Although PCNA has the advantage of being detected in formalin-fixed tissue, immunohistochemical determination of proliferation using this antibody may not accurately reflect the proportion of cells in the growth cycle, for several reasons [13,18]. The number of PCNA immunoreactive nuclei may be altered by different tissue processing techniques [19], and the half-life of the PCNA antigen exceeds the cell cycle time [20]. Consequently, proliferation rates based on PCNA immunohistochemistry may be an inaccurate estimate of the number of proliferating cells. Recently, a new

0022-202X/95/$09.50 • SSDI0022-202X(95)00164-G 

murine monoclonal antibody, MIB-1, has been developed using recombinant portions of the Ki-67 nuclear antigen as an immunogen [21]. MIB-1 recognizes the Ki-67 nuclear antigen on formalin-fixed, paraffin-embedded tissue [22], and thus proliferative studies can now be performed on archival tissue. Our preliminary study of MIB-1 expression in primary cutaneous and metastatic melanomas showed a progressive increase of MIB-1 proliferative rates in primary tumors with deeper levels of invasion and a marked increase in the level of MIB-1 expression in metastatic lesions.** These results are comparable to earlier Ki-67 immunohistochemical studies of melanocytic neoplasms that demonstrated increased proliferation associated with tumor and tumor aggressiveness [11,12,23–26].

In this study, we assessed the proliferative activity of primary thick cutaneous melanomas (tumor thickness 4 mm or greater) using the MIB-1 antibody, comparing the immunohistochemical results with the mitotic index and relating both factors to clinical outcome.

## MATERIALS AND METHODS

**Selection of Patients** Since 1980, data on patients with cutaneous melanoma who are consecutively registered and followed up at the Toronto-Bayview Regional Cancer Centre have been entered in a Medlog data base (Medlog Systems Information Analysis Corporation, Incline Village, NV). The data base documents clinical and pathologic factors in addition to clinical outcome. These factors include demographics, site of the lesion, family history of melanoma, presence or absence of dysplastic nevi, biologic type of melanoma, Clark level of invasion, Breslow vertical height, ulceration, and regression. The data base was searched for all patients with clinical stage I cutaneous melanomas measuring 4 mm or greater in vertical thickness.

**Pathology and Immunohistochemical Methods** The hematoxylin and eosin–stained tissue sections of the primary tumors were reviewed after the patient's initial visit. Breslow measurement of tumor thickness, Clark level, and tumor type were confirmed, and other pathologic features such as ulceration and regression were recorded.

The paraffin blocks of the primary tumors were retrieved from the referring laboratories. One or two representative sections of the deepest areas of each tumor were stained with hematoxylin and eosin. The mitotic index was calculated for each tumor using the method of Schmoeckel and Braun-Falco [8]. Areas of higher mitotic rates were selected by screening at low magnification; the number of mitotic figures was counted in 10 high-power fields (at least 1.5 $mm^2$) at 400× magnification and expressed as mitotic figures/$mm^2$.

An adjacent section 4 $\mu$m thick was used for immunohistochemical study with the murine monoclonal antibody MIB-1 (Immunotech, Boston, MA) by the biotin streptavidin immunoperoxidase technique. Briefly, after blocking of endogenous peroxidase with 3% hydrogen peroxide, the paraffin sections were processed in a 750-W household microwave oven at maximum power for 5 min and then at 50% power for 3 min, three times, to enhance antigen detection. The primary antibody, diluted 1:50 in 0.1% gelatin, was applied for 2 h. The multilink antibody (Dakopatts, Glostrup, Denmark) was applied for 10 min and was followed by the streptavidin complex. For visualization of the MIB-1 antibody, the slides were developed in 0.3% 3′amino 9′ethylcarbazole; Harris hematoxylin was used as a counterstain. Tonsils were used as control tissue, and staining of basal keratinocytes of the epidermis served as an internal control.

**Assessment of MIB-1 Immunoreactivity** The overall pattern of MIB-1 staining of a tumor was categorized as either diffuse if immunoreactive nuclei were evenly distributed throughout the lesion, or focal if MIB-1–positive nuclei were observed in aggregates. At least four fields of 100 tumor cells each (total at least 400 tumor nuclei) per slide were counted at 400× magnification. The number of MIB-1–immunoreactive nuclei in each field was determined and subsequently expressed as a percentage of the total number of tumor nuclei counted. A mean percentage of MIB-1–immunoreactive tumor nuclei was calculated for each lesion. The MIB-1 percentages were scored as low (less than 10%), intermediate (10% to 20%), or high (greater than 20%). MIB-1 proliferative activity was assessed without knowledge of the clinical outcome.

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**Table I. Clinicopathologic Features of Patients With Clinical Stage I Thick Cutaneous Melanomas (Tumor Thickness 4.00 mm or Greater)**

| Feature | Number (N = 64) |
|---|---|
| Sex (male:female) | 46:18 |
| Age at diagnosis (years) | |
| Mean | 54.0 |
| Range | 18.6–87.5 |
| Site | |
| Face | 8 |
| Scalp | 4 |
| Neck | 1 |
| Trunk | 29 |
| Arm | 4 |
| Leg | 13 |
| Foot | 5 |
| Histology | |
| Superficial spreading | 20 |
| Nodular | 31 |
| Lentigo maligna melanoma | 1 |
| Desmoplastic | 3 |
| Acral lentiginous | 2 |
| Not classifiable | 7 |
| Tumor thickness (mm) | |
| Mean | 5.76 |
| Median | 5.00 |
| Range | 4.00–15.00 |
| Follow-up time (years) | |
| Median | 3.8 |
| Range | 0.42–13.6 |
| Outcome[a] | |
| ANED | 23 |
| AWD | 1 |
| DNED | 6 |
| DWD | 30 |
| LTFU | 4 |

[a] ANED, alive with no evidence of disease; AWD, alive with disease; DNED, dead with no evidence of disease; DWD, dead with disease; LTFU, lost to follow-up.

**Statistics** Statistical analyses were performed with the Medlog statistical software program. Comparisons of continuous data were made by the Student t test and of categoric data by the $\chi^2$ test. Survival curves were constructed by the Kaplan-Meier method and compared by the Mantel-Haenszel log-rank test. Multivariate analysis of the association of potential prognostic factors was assessed by Cox regression analysis. To determine the independent effect of MIB-1 reactivity, we included in the multivariate analysis all factors that have been described as predictive of outcome in cutaneous melanoma. These prognostic factors included: sex, site of the primary lesion (both site and BANS (upper back, upper posterior arm, posterior neck, and scalp region [27])), age at diagnosis, histologic subtype, Breslow thickness, Clark level, tumor regression, ulceration, and proliferative activity. Proliferative activity included mitotic index, percentage of MIB-1 immunoreactivity, and pattern of MIB-1 staining. Values of $p < 0.05$ were considered statistically significant.

## RESULTS

Of the 1287 patients in the melanoma data base, 97 were classified as having clinical stage I disease with a primary lesion measuring 4 mm or greater in vertical thickness. Adequate tissue was available for only 64 patients because some cases had been diagnosed more than 10 years before our study, and referring laboratories had discarded the blocks. Comparison of the 33 cases in which no tissue was available and the 64 cases included in the study revealed no significant differences in terms of age at diagnosis, male to female ratio, site of primary lesion, Breslow thickness, Clark level, histologic type of melanoma, presence of ulceration and regression, or last clinical status. Likewise, there was no significant difference observed for overall or cause-specific survival between the groups.

The clinical and pathologic features of the 64 patients in the study group are summarized in **Table I**. Seven tumors could not be subtyped histologically because there was insufficient tissue adja-

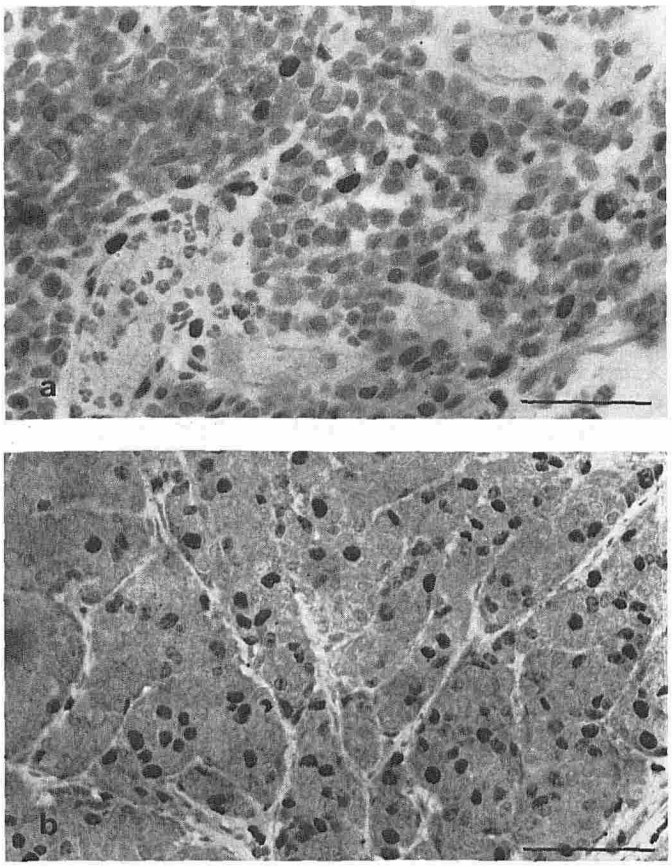


**Figure 1. Clinical stage I cutaneous melanomas 4 mm or greater in vertical thickness.** Demonstration of proliferating tumor cells by MIB-1 immunohistochemical staining of nuclei. *a*) Melanoma with low MIB-1 expression (reactivity less than 10%). *b*) Tumor with high MIB-1 expression (reactivity greater than 20%). *Bars*, 100 μm.

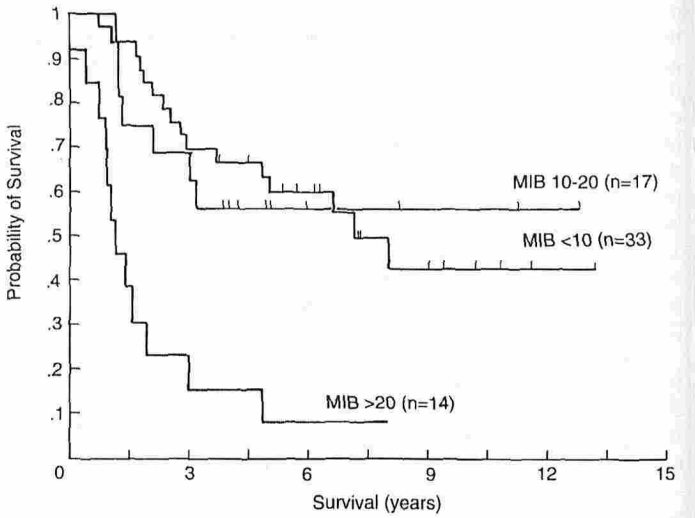


**Figure 2. Cause-specific survival curves in patients with clinical stage I cutaneous melanomas measuring 4 mm or greater based on proliferative activity by MIB-1 immunohistochemistry.** Percentages of MIB-1–reactive tumor nuclei were scored as low (less than 10%), intermediate (10% to 20%), or high (greater than 20%). Comparisons of survival curves were significant for low versus high MIB-1 proliferative rates ($p < 0.0001$) and for intermediate versus high MIB-1 proliferative rates ($p = 0.0025$). There was no significant difference in survival rates between low and intermediate MIB-1 proliferative rates ($p = 0.4957$).

cent to the tumor to assess whether a radial growth phase was present. Patients were followed up for a median of 3.8 years (range 0.42–13.6 years). Thirty-six of the 64 patients developed recurrent disease; of these, 30 died of their disease, one patient is alive with disease, three are alive with no evidence of disease, and two were lost to follow-up. Of the 28 patients without recurrences, 20 are alive with no evidence of disease, six died with no evidence of disease, and two were lost to follow-up. Seven patients were followed for less than 1 year: one with recurrent disease was lost to follow-up and the other six died, five of disease. All of the 13 patients with follow-up for 1–2 years died of melanoma. The remaining 44 patients have been followed up for at least 2 years and of these, 12 died of melanoma and five died of other causes. A further three patients were lost to follow-up, one of whom developed recurrent melanoma before leaving the clinic. At the time of writing, 23 patients were alive with no evidence of disease and one patient was alive with disease.

Tumor nuclei reactive for the MIB-1 antigen were found in both the epidermal and dermal components of melanomas, but the pattern of immunoreactivity was heterogeneous in different lesions. In some melanomas, the reactive nuclei were diffusely distributed throughout the tumors, whereas others exhibited focal aggregates of immunoreactive nuclei. The MIB-1 reactivity scores were as follows: low (less than 10%) in 33 cases, intermediate (10% to 20%) in 17 cases, and high (greater than 20%) in 14 cases **(Fig 1)**. The range of MIB-1 positivity was 0% to 44%. Three melanomas showed no MIB-1 expression despite a reactive internal control.

The correlation of vertical depth with either mitotic rate ($r = 0.157$, $p = 0.2138$) or MIB-1 reactivity ($r = 0.127$, $p = 0.3125$) was not significant. In contrast, the correlation between MIB-1 immunoreactivity and the mitotic index was highly significant ($r = 0.473$, $p < 0.0001$).

There was a lower cause-specific survival rate in those patients with melanomas with high proliferation rates (MIB-1 reactivity greater than 20%) than in patients with tumors of low proliferation (MIB-1 reactivity less than 10%) or intermediate proliferation (MIB-1 reactivity 10% to 20%) **(Fig 2)**. The higher survival rate of patients with low MIB-1–positive tumors compared with those with high MIB-1–reactive melanomas was significant ($p < 0.0001$), as was the comparison between patients with tumors of intermediate and high MIB-1 proliferative rates ($p = 0.0025$). There was no significant difference in survival rates between patients with tumors of low and intermediate MIB-1 scores ($p = 0.4957$). A similar association was demonstrated between MIB-1 proliferative rates and overall survival.

The results of the Cox regression model for overall survival and cause-specific survival are shown in **Table II**. For overall survival, the only variables that attained statistical significance were age at diagnosis ($p = 0.0055$) and MIB-1 immunoreactivity ($p < 0.0001$). For cause-specific survival, the only variable that was statistically significant was MIB-1 immunoreactivity ($p = 0.0002$); vertical depth approached statistical significance ($p = 0.0585$).

## DISCUSSION

Our results show that the proliferation rate determined by MIB-1 immunohistochemistry is inversely correlated with cause-specific and overall survival. Moreover, in the multivariate analysis, MIB-1 proliferative activity was the single most significant prognostic factor in this group of clinical stage I patients with thick cutaneous melanomas. The majority of immunohistochemical studies of proliferation in melanoma have examined proliferation only with respect to other pathologic factors such as vertical thickness [11,23,25,26], Clark level of invasion [14], nuclear size [23,24], or mitotic rate [25,26]. Those few investigations that examined the level of expression of proliferation antigens with reference to patient outcome are not comparable to our study in terms of high-risk stage I patients. Furthermore, these studies have several limitations. First, proliferation has not been evaluated as an inde-

**Table II. Multivariate Analysis of Prognostic Factors With Overall and Cause-Specific Survival in Patients With Clinical Stage I Cutaneous Melanoma (Tumor Thickness 4.00 mm or Greater)**

| Variable | Overall Survival (p Value) | Cause-Specific Survival (p Value) |
|---|---|---|
| *Clinical* | | |
| Age | 0.0055[a] | 0.0797 |
| Sex | 0.9997 | 0.9896 |
| Site | 0.2726 | 0.1358 |
| BANS[b] | 0.2222 | 0.3917 |
| Pathology | | |
| Type | 0.3508 | 0.2724 |
| Level | 0.6881 | 0.2658 |
| Thickness | 0.0700 | 0.0585 |
| Ulceration | 0.7661 | 0.3552 |
| Regression | 0.6970 | 0.2627 |
| Proliferation | | |
| MIB-1% | <0.0001[a] | 0.0002[a] |
| MIB pattern | 0.2929 | 0.4170 |
| Mitotic index | 0.3867 | 0.9123 |

[a] $p < 0.05$; statistically significant.
[b] BANS, upper back, upper posterior arm, posterior neck, scalp region.

pendent prognostic factor in multivariate analysis of clinical and pathologic factors but rather as an attribute in combination with other pathologic features such as the vertical thickness measurement [11] or tumor cell motility [12]. Second, immunohistochemical studies based on PCNA [16,17] or Ki-67 [11,12] are limited by technical considerations [13,18] and a shorter follow-up period than in the current study. Because the mitotic index and detection of proliferation-associated nuclear antigens both measure proliferation, is not surprising that there is a correlation between the factors. However, in contrast to MIB-1 immunoreactivity, the mitotic index is not a statistically significant prognostic factor in multivariate analysis. The failure of the mitotic index to show statistical significance as an independent prognostic attribute in our study may reflect the fact that it recognizes only a fraction of the cells in the proliferative phase as compared with MIB-1, which identifies all proliferating cells from $G_1$ to M.

The presence of focal aggregates of MIB-1–reactive tumor nuclei was not significant in our study; other investigators have suggested that focal aggregates of PCNA reactivity may reflect tumor heterogeneity or clones more likely to invade or metastasize [14].

In this series of thick cutaneous melanomas, the only other clinical or pathologic factor in the multivariate analysis that approached statistical significance was vertical thickness. By restricting the range of thickness of the melanomas studied, we may have decreased the variability of this factor and thus obscured its true value as a prognostic factor. Nonetheless, the results of our study identified a potential prognostic attribute in a select group of stage I patients whose overall prognosis based on tumor thickness is poor. The advantages of studying patients with thick melanomas are that their clinical outcome will be apparent within a few years and that there is a subgroup of patients with a better prognosis. It would also be important to study low-level melanomas to identify those few patients with a poor prognosis. Large numbers of cases would be required, however, to show statistical significance; the follow-up period would have to be longer, and the volume of melanoma tissue would not necessarily be adequate to perform such a study.

As with all retrospective studies, this investigation has some limitations. First, not all paraffin tissue blocks could be retrieved; however, there was no significant difference in survival or in clinical and pathologic features between the patients studied and those whose tumors were not available for MIB-1 immunohistochemistry. Second, although the majority of patients were referred to the Melanoma Clinic within 5 weeks of diagnosis, some patients had later referrals; however, there was no significant difference in survival between these groups. Therefore, although this study is not a complete assessment of all 97 patients and is not a true inception cohort among the 64 patients studied, we believe that any possible selection bias is small and is unlikely to alter the importance of our observations.

We have demonstrated that MIB-1 proliferative activity is an independent prognostic factor in melanomas measuring 4 mm or greater in thickness. Thus, a subset of patients can be identified who are at lower risk of recurrent disease than is otherwise indicated by measurement of the vertical thickness of their tumors. This knowledge will be important for assessment of new treatment modalities for thick melanomas.

*This work was supported by a grant from the Bassett-Falk Cancer Research Foundation. We are grateful for the expert technical assistance of M. Shackelton and K. Kwok. We also acknowledge N. Lassam and G. DeBoer for their helpful comments.*

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